The Role of RNA Polymerase and Negative Cell Cycle Regulators in Facilitating Stringent Arrest in *Escherichia coli*

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Abstract

Previous research has implicated negative regulators of cell cycle in *Escherichia coli*, in particular the SeqA protein, in the stringent response. The stringent response is caused by amino acid starvation, and results in a complete cessation of initiation of replication at the origin of replication, *oriC*, causing a percentage of a population of cells to arrest with 1N chromosome equivalents. We believe that long-term stable binding of SeqA to hemi-methylated GATC sites at *oriC* during stringent response is the cause of this arrest. Mutations that affect the capacity of SeqA to bind at the origin also remove the ability of the cells to arrest at 1N. Deletion of *seqA* from the *E. coli* genome causes deficiency in stringent arrest. Deletion of *dam* methylase - which leads to unmethylated DNA, to which SeqA does not bind – causes the same effect. Over-expression of *dam*, which causes premature full methylation at newly replicated GATC sites, also disrupts stringent arrest. Mutation of 9 GATC sites in *oriC* to GTTC also partially disturbs stringent arrest.

We are also interested in the role of RNA polymerase in facilitating the stringent response. It is known that widespread changes in transcriptional patterns occur during stringent arrest, and we are in possession of strains mutant in the RNA polymerase beta subunit that mimic stringent transcriptional patterns in the absence of amino acid starvation. The *rpoB* mutants display partial 1N and 2N arrest without amino acid starvation. In addition, the mutations described above (*seqA, dam, oriCm3*) that disrupt stringent arrest also suppress cell cycle arrest phenotypes in *rpoB* mutants.
**Introduction**

**SeqA and Dam Regulate Replication and Division in *E. coli***:

Recent studies have implicated SeqA in having a key role in the cell cycle of *E. coli*. *SeqA* is a 500 bp gene that encodes a protein that is involved in both initiation of replication and chromosome segregation. Its role in control of replication initiation is much more well defined.

Replication of the bacterial chromosome begins at the origin of replication, *oriC*. At the time of initiation, both strands of DNA are methylated at adenosine residues in GATC sites. However, just after replication begins, each new double-stranded *oriC* site is only hemi-methylated; the template strand remains in its methylated state while the newly synthesized strand is unmethylated (Campbell and Kleckner, 1990). SeqA binds preferentially to hemi-methylated GATC sequences, which exist non-exclusively within *oriC*, and sequesters the origin from initiation factors, preventing further replication (see Figure 1) (Campbell and Kleckner, 1990; Sutera et al, 2006).

![Diagram](image)  

**Figure 1.** Shown is the mechanism by which SeqA binding sites are formed just after primary initiation. New, unmethylated strands are synthesized from methylated template.
The period of time immediately after replication initiation, during which SeqA is bound to oriC and during which new replication forks can not be formed, is known as the eclipse period (Freiesleben, Krekling, Hansen, Løbner-Olesen 2000). The eclipse period does not last the duration of the elongation period, as rapidly-growing cells can initiate secondary replication events before primary replication and division is complete. It has been shown that SeqA binds at the origin of replication and that the timing of the eclipse period is correlated with this interaction (Lu, et al, 1994). The role of SeqA in the eclipse period is to oversee proper timing of initiation with respect to replication, and ensure that all oriC sites on sister chromosomes initiate secondary replication simultaneously (Sutera et al 2006). The eclipse period terminates when SeqA is removed from oriC, though the mechanism of SeqA removal is unknown. This allows Dam methylase to methylate newly replicated DNA strands at GATC sites, lowering the affinity of SeqA for these binding sites and preventing it from further sequestering these sites from initiation factors (Sutera et al 2006).

**Phenotypes Associated with Cell Cycle Defects:**

It has been shown that the eclipse period is dramatically shortened in seqA mutants (Olsson, 2003), as seqA- cells exhibit rampant over-initiation due to loss of SeqA-oriC interaction (see Figure 2). Furthermore, over-expression of SeqA in wild-type extends the eclipse period by prolonging the amount of time during which SeqA is bound to hemimethylated GATC sites at oriC (Bach, Krekling, Skarstad, 2003). Conversely, over-expression of Dam causes mild over-initiation, presumably by methylating nascent GATC sites at oriC before SeqA can bind (see Figure 2), thus shortening the eclipse period (unpublished data). Deletion of dam causes GATC sites to remain unmethylated at all
times, completely eliminating all SeqA binding sites (see Figure 2) and causing many of the same effects as seqA deletion (Sutera et al, 2006).

It has been shown that seqA and dam mutants are sensitive to DNA-damaging agents such as ultra-violet radiation (UV), hydroxyurea (HU), and AZT compared to wild-type (Sutera et al, 2006; unpublished data). Treatments such as these cause minor chromosomal damage, which leads to stalled replication forks at damage sites (reviewed in Marinas KJ). The sensitivity of seqA and dam mutants to these agents is likely due to the loss of damage-dependent replication restraint. Premature initiation preceding damage repair in these strains would cause secondary replication forks to converge on primary forks stalled at damage sites causing large chromosomal losses. This results in widespread chromosome instability and ultimately cell death (Sutera et al, 2006). Strains deficient in both SeqA and RecA-dependent DNA repair and are much more sensitive to UV radiation and HU than either single mutant, showing that each protein represents an independent damage-response pathway. In addition, seqA dam double mutants show sensitivity identical to the seqA mutant alone, showing that SeqA and Dam are part of the same damage-survival mechanism (Sutera et al, 2006).

**Figure 2.** Shown are diagrams of oriC just after initiation of replication in different mutants *left:* deletion of seqA causes over-replication, as SeqA does not bind; *middle:* deletion of dam leaves DNA without methylation, making SeqA unable to bind; *right:* over-expression of dam can fully methylate prematurely, hindering SeqA binding.
In the absence of SeqA binding, oriC is not sequestered following primary initiation of replication, and subsequent rounds of replication are quickly initiated in a manner dependant on DnaA, the initiation factor (Sutera et al 2006). One of the major roles of SeqA is to prevent contact between oriC and DnaA, thereby preventing initiation as long as SeqA is bound. Strains with defects in dnaA show poor growth and display phenotypes consistent with failure to initiate replication. However, mutations in dnaA also substantially suppress seqA mutants’ sensitivity to AZT and HU (Sutera et al 2006). This suggests that the over-initiation effect in seqA mutants and under-initiation as consequence of dnaA co-suppress each other, and further displays the role of SeqA in controlling initiation. This is also very useful, because it provides a way to study other aberrant phenotypes in seqA null mutants that do not over-initiate.

It has also been shown that SeqA has a very important role in regulating initiation of replication under stressful conditions. Null seqA mutants grow poorly and are more sensitive to DNA damage while growing in rich media, which stimulates quick proliferation and increases frequency of secondary initiation. Growth in minimal media, which lowers the frequency of replication initiation in a SeqA-independent manner, partially suppresses the requirement for SeqA-dependant initiation control (Sutera et al 2006). However, growth on minimal-AZT medium provides a completely different result. Strains with wild-type seqA (seqA+) grew very poorly on AZT minimal media, suggesting a damage-dependant arrest mechanism is at work (Sutera et al 2006). The seqA− dnaA+ strain also plated very poorly as a result of over-replication in the presence of DNA damage. Surprisingly, the seqA− dnaA446 double mutant plated thousand-fold better than wild-type on AZT-minimal media (Sutera et al 2006). This suggests that replication is actively
inhibited in a SeqA-dependant manner in response to DNA damage, with the \textit{seqA dnaA} double mutant released from this restraint.

**The Stringent Response:**

Given the importance of SeqA in initiation restraint in response to DNA damage, we are investigating the importance of SeqA in the stringent response. The stringent response is the bacterial response to amino acid starvation. Stringent response activation originates at the ribosome. A small percentage of ribosomes have bound to them the protein RelA, binding of an uncharged tRNA to the ribosome, which happens frequently during amino acid starvation, causes RelA to be released into the cytoplasm. Free RelA, along with other regulating factors, phosphorylates GDP and GTP, up-regulating intracellular concentrations of Guanosine-5'-biphosphate-3'-biphosphate, also known as ppGpp (unpublished data, Schreiber et al 1995).

High levels of ppGpp cause inhibition of initiation at \textit{oriC}, arresting the cell cycle in cells which have not yet started replication (Schreiber et al 1995). Cells that are in the middle of replication do not stop replicating, though secondary initiation is blocked. Instead, induction of the stringent response inhibits chromosome segregation and cell division, blocking the cell cycle after completion of replication (unpublished data, Schreiber et al 1995). As such, the stringent response causes a bi-phasic arrest – arrested cell populations will have either 1 or 2 chromosome equivalents (termed 1N and 2N), having been blocked at either initiation or segregation (unpublished data, Schreiber et al 1995).

ppGpp changes transcriptional patterns by directly binding to RNA polymerase. This binding is largely dependent on the protein DksA, which stabilizes the RNA
polymerase--ppGpp complex (Paul BJ et al, 2004). This complex changes transcription patterns by altering the stability of open complex formation at certain promoters (reviewed in Magnusson et al 2005). This change in stability diminishes the ability of RNA polymerase to make mRNA transcripts of genes under “stringently controlled” promoters. The best examples of this effect are rRNA genes, whose promoters form intrinsically unstable open complexes with RNA polymerase. Transcription at these promoters is therefore particularly sensitive to further destabilization by ppGpp (reviewed in Magnusson et al 2005). Binding of ppGpp and DksA to RNA polymerase also stabilizes formation of open complexes at some amino acid promoters (Paul, BJ, Berkmen MB, Gourse RL 2005). In this way, ppGpp and DksA serve to downregulate transcription of genes promoting growth and proliferation and upregulate, among other things, amino acid biosynthesis and stress survival (reviewed in Magnusson et al 2005). ppGpp-bound RNA polymerase also seems to have an essential role in facilitating 1N and 2N arrest, as null mutants in dksA are deficient in this arrest (unpublished data).

The stringent response can be induced in *E. coli* by incubating cycling cells with 1mg/mL DL-serine hydroxamate (Sigma). Serine hydroxamate (SHX, see Figure 3) is a serine analogue which inhibits seryl-tRNA synthetase. Cells grown in the presence of serine hydroxamate are unable to synthesize serine-bound tRNA, which causes accumulation of uncharged tRNAs. Cells attempting to grow in the presence of SHX will be arrested for as long as the drug is present in culture. Removal of SHX from liquid culture causes arrested cells to divide and resume replication once more (unpublished data).

**Figure 3.**

![Serine Hydroxamate](image)

![Serine](image)
Detection of Stringent Arrest Phenotypes using Flow Cytometry

The DNA content in stringent cells is best visualized by using flow cytometry (FACS). Culture can be fed into a flow cytometer, which can count and score individual cells for size, complexity, and fluorescence. If cellular DNA is first stained by treating cells with PicoGreen dsDNA reagent (Invitrogen), FACS can give a histogram showing the DNA content distribution in the population of cells in culture. To examine arrest of replication and division after induction of the stringent response, flow cytometry can be used to measure the DNA content of wild-type cells growing in the presence of SHX.

Analysis of DNA content in wild-type cultures grown in these conditions for extended periods of time ("run-out" conditions, 90-120 minutes) shows a large population arrested with two chromosome equivalents. A much smaller population is also seen with one chromosome equivalent (see Figure 4). This shows that, under stringent response conditions, cells are blocked at both initiation of replication and cell division. Control experiments show that, without stringent arrest, most cells in culture have between 1-2 chromosome equivalents, implying that replication is in progress in the majority of normally growing cells. This accounts for the larger population of cells with 2N chromosomes compared to 1N, and shows that continuation of replication already in progress is not blocked.

Figure 4. Flow cytometry gives a DNA histogram of a wild-type culture with and without SHX.
in response to amino acid starvation (unpublished data).

ΔseqA mutants are deficient in stringent arrest. Flow cytometry analysis of these mutants in minimal media culture shows a broad distribution of chromosomal DNA, with cells containing anywhere between 1 and 6 chromosomes (unpublished data). This is consistent with the hypothesis that seqA mutants have little control over timing of replication initiation. However, after incubation with serine hydroxamate, no arrest is observed – the cells continue to over-replicate and show the same chromosome equivalent distribution as before incubation with SHX (unpublished data). This shows that SeqA has an essential role in stringent response arrest. Similar results are seen in dam mutants (unpublished data).

Summary of Goals and Techniques:

Our goal is to elucidate the mechanism by which the stringent response causes the arrest of replication initiation. Thus far, one of the biggest questions in this area of research has been the link between ppGpp and 1N arrest. How does accumulation of intracellular ppGpp lead to the block at initiation of replication? Our hypothesis is that RNA polymerase is this link. As discussed above, it is known that ppGpp binds to RNA polymerase, changing transcriptional patterns. According to our model, this change in transcription, alone, is both sufficient and essential to arrest replication and division.

To study the validity of this model, we have obtained two mutants that have mutations in RNA polymerase. These mutants, denoted rpoB3443 and rpoB3370, have missense mutations in the beta subunit of RNA polymerase. They exhibit resistance to the antibiotic rifampicin (rif), which normally acts by binding at the site of the mutation and preventing transcription (Zhou and Jin, 1998). They were first discovered in a rif-
resistance screen and it was found that the mutant RNA polymerase had highly decreased rate of transcription from stringent promoters, but not from non-stringent promoters (Zhou and Jin, 1998). Therefore, these strains have all the traits of cells showing stringent transcriptional patterns, mimicking the RNAP-DksA-ppGpp complex. The strains exhibit poor growth, with rpoB3370 being more severe in this respect (unpublished data).

We plan on further characterizing these rpoB mutants in an effort to see to what extent stringent transcription affects cell cycle arrest. We plan on determining the connection between SeqA and RNA polymerase by moving mutations in cell cycle regulation genes (∆seqA, dam::Tn5) and stringent response control genes (dksA::Kn) into rpoB mutant backgrounds. In addition, we are in possession of a strain that with the genotype oriCm3 which has been mutated at oriC, changing roughly half of the GATC sites to GTTC (Bach and Skarstad, 2004). This strain could prove useful if put in an rpoB mutant background. We also plan on over-expressing SeqA and Dam in wild-type and rpoB mutant backgrounds, in an attempt to see how it affects stringent arrest. Over-expression of SeqA in wild-type could perhaps also delay release from stringent response. Using flow cytometry, we will gather data by visualizing DNA content distribution in homogenous cell cultures with and without serine hydroxamate. By genetically manipulating the different factors believed to be involved in stringent arrest and initiation of replication (SeqA, Dam, DnaA, DksA, RpoB) and viewing DNA content in populations of these mutants, we hope to gather evidence for our model.
**Materials and Methods**

**Bacterial Strains, Plasmids, Antibiotics, Media:**

Strains constructed by P1 transduction (Miller JH, 1992) and electroporation transformation (Miller JF et al, 1988) and are listed in table 1. All strains constructed in MG1655 background except for boldened strains which have backgrounds as noted. *BtuB::Tn10* is an innocuous insertion mutation used as a marker for *rpoB*.

**Table 1: Strains Constructed in MG1655 Background**

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<th>Plasmid:</th>
<th>Marker:</th>
<th>Origin or Reference</th>
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<td></td>
<td>Kn&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
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<td><em>seqA::Cm::FRT</em></td>
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<td></td>
<td>Kn&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Nick Cozzarelli; W3110 background***</td>
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Note: all strains with genotypes rpoB3443 or rpoB3370 have rif resistance in addition to other drug markers

*MG1655 has the genotype F- rph-1

**KL227 has the genotype metB1 relA1

***W3110 has the genotype (lambda- F- tN(rrnD-rrnE)1 rph-1)
**Table 2: Plasmids**

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</tr>
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<tr>
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<td>bla (Ap&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>expression clone of dam under arabinose promoter</td>
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The pbad18 system was used to express cloned genes directly from the plasmid. Cells harboring pbad18 will over-express genes cloned under the pbad promoter when grown in media containing arabinose. Cells grown in media containing glucose are strongly inhibited from expressing genes cloned under the pbad promoter. Cells grown in media containing an alternate carbon source, such as rhamnose, will exhibit "leaky" expression. For a detailed description of the pbad system, see Guzman et al., 1995. Genes were cloned into pbad18 using Gateway® Technology (Invitrogen).

Strains were grown in LB broth containing 1% (w/V) tryptone, 0.5% (w/V) ycast extract, 0.5% (w/V) sodium chloride, and 50μg/mL thymine all in ddH<sub>2</sub>O. Plate media also contained 1.5% (w/V) agar. LCG media, in which cultures were grown in preparation for P1 lysates and transductions, contained LB media supplemented with 0.1% (w/V) glucose and 2mM CaCl<sub>2</sub>. LCG plate media also contained 1% (w/v) agar. LCG top agar used for harvesting lysates contained 0.7% (w/V) agar. Culture preparations for FACS were done exclusively in M9 minimal media containing 20% (V/V) M9 salts (.24M Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, .11M KH<sub>2</sub>PO<sub>4</sub>, .04M NaCl, .1M NH<sub>4</sub>Cl), 2mM MgSO<sub>4</sub>, .4% (w/V) carbon source (glucose, rhamnose, or arabinose, as needed), .1mM CaCl<sub>2</sub>, and 2% (V/V) case amino acid (CAA)
(Bacto) solution in ddH₂O. Cell pellets obtained during FACS preparation were resuspended in 1mL of 1x phosphate buffered saline (PBS; 14M NaCl, 2.7mM KCl, 10.1mM Na₂HPO₄, .18mM KH₂PO₄, pH 7.4). The following antibiotic concentrations were used as needed in all media: 100µg/mL ampicillin (Ap), 30µg/mL chloramphenicol (Cm), 60µg/mL kanamycin (Kn), 15 µg/mL tetracycline (Tc), 100µg/mL rifampicin (rif).

P1 Lysates and Transductions

P1 lysates were made as follows:

1. Prepare a 5mL LCG standing overnight culture at 37°C of the strain harboring the mutation to be transduced
2. The next day, warm 7 LCG plates at 37°C
3. Aliquot 200µL of standing culture into 7 small glass test tubes
4. Add 10µL of P1 phage stock into the tube 1 and vortex
5. Take 200µL of culture from tube 1, add to tube 2, and vortex
6. Take 200µL of culture from tube 2, add to tube 3, and vortex
7. Take 200µL of culture from tube 3, add to tube 4, and vortex
8. Take 200µL of culture from tube 3, add to tube 4, and vortex
9. Take 200µL of culture from tube 4, add to tube 5, and vortex
10. Take 200µL of culture from tube 5, add to tube 6, and vortex
11. Tube 7 gets no phage and is used as a control
12. Incubate all 7 tubes at 37°C for 20 minutes
13. Remove the tubes from the incubator and add 2.5mL of molten LCG top agar to each tube and vortex
14. Spread top agar from each tube onto a separate LCG plate and incubate at 37°C for at least 8 hours

15. Choose a plate that has many small plaques that are closely spaced and running into each other

16. Using a metal spatula, scrape off the top agar into a 30mL corex glass centrifuge tube, and add 2mL LB and 0.5mL chloroform

17. Vortex hard for at least 1 minute and let sit at room temperature for 10 minutes

18. Spin in a centrifuge at 7000rpm for 10 minutes

19. Remove the upper LB layer using a pasteur pipette and store in a glass vial at 4°C

**Transductions were performed as follows:**

1. Prepare a standing overnight LCG culture of the strain to be transduced into

2. Aliquot 1mL of standing culture into 3 microcentrifuge tubes and pellet the cells by spinning at 13,000rpm for 3 minutes

3. Pour off the media and resuspend the pellet in 100μL fresh LCG

4. Add 5μL P1 lysate of the desired mutation to the first tube and 25μL lysate to the second tube. The third tube gets no phage and will act as a control.

5. Incubate all three tubes at 37°C for 30-40 minutes

6. Remove the tubes and add 100μL 1M sodium citrate to each tube

7. Spread each of the mixtures on plates selective for the mutation to be transduced. Mutations in *rpoB* and *dnaA* are both linked to TcR while *dksA* and *dam* mutations are KnR. The *seqA* deletion is CmR.

8. Streak out individual isolates from the transduction plates to freeze away only if nothing grows on the control plates. In most cases, gained antibiotic resistance is
enough to confirm successful transduction. However, \( rpoB \) mutations are linked to 
\( btuB::Tn10 (\text{Tc}^R) \) and co-transduce with \( btuB \) at a rate of about 60%. When
selecting \( rpoB \) transductants, select the smallest colonies and patch streak them on
\( rif \) plates to ensure that they are \( rif \) resistant.

**Plasmid Purification:**

All plasmid purifications were done using a Sigma GenElute\textsuperscript{TM} Plasmid Miniprep Kit and
checked for yield via agarose gel electrophoresis.

**Electroporation Transformations:**

*Competent cells* were made as follows

1. Streak out the strain to be transformed on an agar plate containing appropriate
antibiotics, and grow overnight at 37\(^\circ\)C

2. Using a sterile stick, scrape cells from the plate into a microcentrifuge tube

3. Resuspend cells in 1mL of cold, sterile H\( _2 \)O

4. Pellet the cells by centrifuging at 13,000rpm for 3-5 minutes

5. Pour off the H\( _2 \)O and repeat steps 3 and 4

6. Resuspend in 80-100\( \mu \)L cold, sterile H\( _2 \)O and transform immediately

*Note: this technique is used to quickly make cells for transformations not requiring high
efficiency. High efficiency transformations require competent cells made with a more
meticulous technique.

*Electroporation transformations were performed as follows

1. Aliquot 1mL of LB media into 2 test tubes

2. Aliquot 40\( \mu \)L competent cells into two microcentrifuge tubes
3. Drop dialyze 3-4μL purified plasmid DNA for 10 minutes

4. Add 2μL drop dialyzed plasmid to one of the tubes containing competent cells

5. Add mixture to an electroporation cuvette and pulse with 2.5V and 200W for 1s using a BIO-RAD Gene pulser™

6. Immediately pour 1mL LB from the test tube into the cuvette, mix by pipetting up and down, and return to the test tube

7. Repeat steps 5 and 6 using the 2nd tube of cells, to which no DNA was added

8. Incubate test tubes shaking at 37°C for 1 hour

9. Spread 200μL from each tube on LB plates containing antibiotic selective for the plasmid used to transform, and incubate overnight at 37°C

10. Streak out individual colonies to freeze only if there are no colonies growing on the control plate. If the transformation was done in an rpoB mutant, also streak isolates on rif plates to ensure that suppression of rpoB did not occur.

Flow Cytometry:

*Samples for visualizing stringent response with flow cytometry were prepared as follows:*

1. Prepare an overnight standing culture at 37°C in M9 glucose media of the appropriate strains. STL8298 should be grown at 30°C and STL8693 should be grown at 34°C. Strains harboring pbad18-derived expression clones should be grown overnight in M9 rhamnose media.

2. The following day, dilute 100μL of culture into 10mL M9 media in 15ml conicals and grow shaking at 37°C for 1 hour. STL8298 should be grown at 30°C and STL8693 should be grown at 34°C. Strains harboring pbad18-derived expression clones should be diluted into glucose or arabinose media.
3. Dissolve serine hydroxamate in ddH₂O and mix until completely dissolved.
   Sterilize by filtration.

4. Divide cultures in half by aliquoting 5 mL of each culture into two different 15mL conicals. Add serine hydroxamate solution to one of each of these conicals to a final concentration of 1mg/mL. The other half of the conicals get no SHX and are used as controls.

5. Incubate all 15mL conicals spinning at 37°C for 90-120 minutes. STL8298 should be grown at 30°C and STL8693 should be grown at 34°C.

6. Suspend 1mL of each culture in 9mL cold 70% ethanol in a 15mL conical and store overnight at 4°C. Samples can be kept cold in ethanol for at least two weeks.

Samples for visualizing stringent response release were prepared as follows:

1. Follow steps 1-6 above

2. Pellet cells by centrifuging each conical at 4000rpm

3. Decant media and resuspend cell pellets in fresh M9 media. Fresh media should contain sugar and antibiotic corresponding to the media that was just decanted.

4. Repeat steps 2-3

7. Incubate all conicals shaking at 37°C. Every 30 minutes, remove 1mL of each culture and add it to 9mL cold 70% ethanol in a 15mL conical and store overnight at 4°C. Terminate the experiment after 120 minutes. Samples can be kept cold in ethanol for at least two weeks.
Cell samples were stained as follows

1. Centrifuge samples in ethanol at 4000 rpm for 10 minutes. Cell pellets should be visible.

2. Decant all ethanol off the cell pellets. Invert the tubes and let sit for 10 minutes to let residual ethanol evaporate.

3. Resuspend each cell pellet in 1 mL PBS buffer and move each sample into a Falcon 5 mL collection tube.

Steps 4-5 should be done in the dark

4. Thaw PicoGreen dye. Make a 1:100 dilution of PicoGreen in 25% DMSO.

5. Add 200 μL PicoGreen/DMSO to each sample. Cover and leave at room temperature (in darkness) for 30 min - 3 hours. Samples are then ready to be run through the flow cytometer.
Results:
Shown in Figure 1 is the DNA content profile for both rpoB alleles compared to wild type. These rpoB mutants show a partial stringent arrest phenotype even in the absence of SHX. They are fully arrested in the presence of SHX, showing that their ability to bind ppGpp remains intact. They also show abnormally large 1N peaks in both conditions, and throw off large numbers of anucleates\(^1\), suggesting that they are trying to divide in the absence of replication. SHX appears to cause anucleation even in wild-type populations.

Figure 1:

\(^{1}\) Numbers in the histograms represent the percentage of total events that lie within the interval shown. Events lying on the y-axis represent anucleate cells and incomplete stainings.
Figure 2 shows that *dksA* null mutants are deficient in stringent cell cycle arrest, and that *rpoB3443* and *rpoB3370* suppress this phenotype. These strains no longer require DksA to stabilize ppGpp binding, suggesting that weak ppGpp–RNAP interaction is sufficient to cause full stringent arrest in *rpoB* mutants that already have stringent transcription patterns. This underscores the importance of transcriptional changes in stringent response.

**Figure 2:**

- **no SHX**
  - *dksA::Kn*
  - *rpoB3443 dksA::Kn*
  - *rpoB3370 dksA::Kn*

- **SHX**
  - *dksA::Kn*
  - *rpoB3443 dksA::Kn*
  - *rpoB3370 dksA::Kn*
We next wanted to show a connection between stringent arrest and SeqA function at oriC by introducing a null mutation in dam. Figure 3 shows that all strains harboring the dam::Tn5 null mutation are deficient in SHX-induced stringent arrest, and that the mutation suppresses the stringent phenotypes brought on by rpoB mutation. This shows that Dam is downstream of RNAP in the pathway between ppGpp and replication arrest.

Figure 3:

no SHX

SHX
We hypothesized that over-expression of Dam might also disrupt SeqA function by prematurely methylating nascent DNA at oriC. Figure 4 shows that over-expression of Dam from the pbad18 arabinose promoter causes wild-type cells to exhibit no cell cycle arrest when incubated in SHX. It also shows that over-expression of Dam in an rpoB3443 background disrupts its stringent phenotypes.

**Figure 4:**

- **no SHX**
  - Wild-type
  - Wild-type + pbad18 arabinose

- **SHX**
  - 57.3
  - 50.7

- **rpoB3443**
  - 24.0
  - 10.9
We wanted to show that functional SeqA protein is necessary for stringent arrest.

Figure 5 shows that seqA deletion mutants are deficient in stringent arrest, and that the seqA deletion suppresses arrest caused by the rpoB mutations.

**Figure 5:**

![Graphs showing SeqA and RpoB effects with and without SHX](image)

- **no SHX**
  - brtB::Tn10
  - seqA::Cm::FRT

- **SHX**
  - 92
  - 51.7
  - 78.2
  - 56
  - 42.5
  - 9
Disrupting SeqA binding sites in oriC also causes deficiency in stringent arrest.

Figure 6 shows that a strain with half the GATC sites in oriC mutated to GTTC displays mild over-replication and is partially deficient in stringent cell cycle arrest. OriCm3 also appears to have a partial suppressor effect on rpoB3443 and rpoB3370 without SHX, but only slightly disrupts SHX-induced stringent arrest in these mutants.

Figure 6:
We hypothesized that over-expression of SeqA might interfere with re-initiation and division after release from stringent response. Figure 7 shows that over-expression of SeqA in wild-type from the pbad18 arabinose promoter does not delay release from stringent response. Time points are taken from the time of removal of SHX from culture. Cells overexpressing SeqA do not appear to be arrested at 1N and 2N for longer than the control.

**Figure 7:**

<table>
<thead>
<tr>
<th>Time</th>
<th>Wild-type +pbad18 arabinose</th>
<th>Wild-type +pbad18seqA arabinose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0'</td>
<td><img src="image1" alt="Graph" /></td>
<td><img src="image2" alt="Graph" /></td>
</tr>
<tr>
<td>30'</td>
<td><img src="image3" alt="Graph" /></td>
<td><img src="image4" alt="Graph" /></td>
</tr>
<tr>
<td>60'</td>
<td><img src="image5" alt="Graph" /></td>
<td><img src="image6" alt="Graph" /></td>
</tr>
</tbody>
</table>
Discussion:

Characterization of stringency of the rpoB3443 and rpoB3370 alleles:

Zhou and Jin characterized these two rpoB alleles as being "stringent" in that they cause RNAP to have decreased ability in transcribing from stringently controlled promoters. From figure 1, it is clear that they also exhibit partial stringent arrest even in the absence of any stringent response induction. Of particular interest is the fact that, in stark contrast to wild-type, the vast majority of rpoB mutants are arrested at 1N in the presence of SHX. This suggests that, since the addition of SHX does not cause inhibition of replication in progress, large populations of cells in culture have failed to initiate replication. This hypothesis is supported by the data in figure 1, as 1N peaks are visible for the two rpoB mutants even without induction of arrest. Obviously, this is not a complete effect, because the strains are viable. However, the decreased viability of the mutants is likely due, at least in part, to the fact that they are inefficient at initiating replication.

Is the partial arrest caused by rpoB3443 and rpoB3370 a product of partial stringent response activation, or simply a side effect of the mutation, caused through a different mechanism? If the "stringent" change in transcription in the rpoB mutants is not the cause of the cell cycle block, then deletion of dksA in an rpoB background would eliminate complete ppGpp-mediated arrest, as it does in wild-type. However, Figure 2 shows that this is not the case. Even without functional DksA protein, the rpoB mutants exhibit partial arrest in minimal media indicating that "stringent" RNA polymerase is sufficient to cause partial replication arrest. The results shown in Figure 2 also raise an interesting question, since, even without DksA, it appears that the mutant RNA polymerases can still bind ppGpp, causing full arrest. This could be due to a strengthening of RpoB-ppGpp interactions due to the mutation or it could be that weaker RpoB-ppGpp interactions are
sufficient to cause full arrest in the presence of the mutation. Given that two different mutations give the same result, the latter of the two scenarios is more likely.

**Role of SeqA in Stringent Cell Cycle Arrest:**

Is inefficiency in replication initiation in the *rpoB* mutants caused by prolonged binding of SeqA to *oriC*? Data shows that mutations known to alter the ability of SeqA to bind to *oriC* cause partial or complete loss of stringent arrest. This was demonstrated in a number of ways.

Figure 3 illustrates that a mutant with no Dam function is completely deficient in stringent arrest, showing no separation of 1N and 2N peaks after incubation with SHX. Deletion of *dam* causes DNA to remain unmethylated at all times, severely lowering the affinity of SeqA for its binding site. Under these conditions, it would not be able to effectively sequester origins under normal growth conditions. Figure 3 also shows that a null mutation in *dam* suppresses the effects of the *rpoB* mutations. This effectively puts Dam downstream of RNA polymerase in the stringent arrest pathway, and further indicates that stringent arrest is not possible without DNA methylation, even in the presence of stringent polymerase. The *dam* mutant's lack of any sort of stringent response, even in *rpoB* backgrounds, further implicates SeqA as having a pivotal role in cell cycle arrest.

Figure 4 shows the effects of over-expression of *dam* in both wild-type and *rpoB* backgrounds. Over-expression of *dam* would cause new hemi-methylated GATC sites to be fully methylated prematurely, before SeqA is able to bind. This would lower SeqA affinity for its binding site somewhat, though SeqA binds to fully methylated DNA with higher affinity that it does unmethylated DNA. For this reason, we would expect any over-replication phenotype to be milder than in the strain with no *dam* function. Indeed, this is
exactly what is displayed in figure 4. In a wild-type background, mild over-initiation is observed and severe deficiency in stringent arrest is apparent. In addition, over-expression of *dam* in *rpoB3443* background causes partial suppression of the *rpoB* phenotypes, as no separation of peaks is observed in the absence of SHX. Even with SHX, *dam* over-expression appears to decrease the size of the 1N peak, indicating that *dam* over-expression disrupts 1N arrest.

Outright deletion of *segA* caused many of the same effects as *dam* deletion and over-expression. As expected, *segA* deletion also fully suppresses the stringent effects in *rpoB3443* and *rpoB3370*, as is shown in figure 5 and caused complete deficiency in SHX-induced arrest in all backgrounds, further cementing the argument that SeqA function is necessary for stringent inhibition of replication. Unfortunately, deletions in *segA* are very poorly viable and accumulate suppressors. In addition, it is difficult to draw any concrete conclusions from DNA histograms of strains with deleted *segA*, because *segA* strains exhibit extremely severe over-replication. Even if replication and division were to be blocked by addition of SHX in such a strain, a DNA histogram would still show a broad distribution as a result of over-initiation and asynchronous replication prior to addition of SHX. For this reason, the *segA* deletion is much easier to work with in a *dnaA46* background, which suppresses the over-initiation phenotype and improves viability. We attempted to run experiments with the *segA* deletion mutation in a *dnaA46* background, but were unsuccessful, though it would be useful to re-attempt this experiment in the future.

More proof can be seen that sequestration at *oriC* is needed to facilitate stringent arrest in figure 7. Strains with the *oriCm3* genotype have a partially mutated origin of replication, with about half of the SeqA binding sites mutated. According to data, this strain shows a slight over-initiation phenotype and a very slight deficiency in stringent
arrest. However, \textit{oriCm3} has a clear partial suppression effect on \textit{rpoB3443} and \textit{rpoB3370}, and is consistent with the other data obtained in this experiment.

**Stringent Arrest Release:**

The final figure shows that populations of cells can be rescued from stringent arrest simply by removing them from SHX culture. It was hypothesized that if SeqA was responsible for blocking initiation at \textit{oriC} during replication, then over-expression of SeqA might cause stringent arrest to persist even once the influence of SHX was removed. Figure 8 shows that this is not at all the case; it is not possible to prolong stringent arrest by over-expressing SeqA. However, this result still fits our model, as it might suggest that SeqA is actively removed from \textit{oriC} upon release from stringent response and perhaps also at the end of the eclipse period. Active removal of SeqA would make over-expression of SeqA much less likely to have a visible effect.

**Mechanisms for transcription-mediated arrest:**

We have presented strong evidence that stable, long-term binding of SeqA to \textit{oriC} during the stringent response is the cause of inhibition of replication initiation. Still elusive, however, is the connection between ppGpp-mediated transcriptional changes and stabilized binding of SeqA. There are different mechanisms by which this could be accomplished. In one proposed model, transcription at the origin during normal growth would serve to actively remove SeqA, thus allowing full methylation by Dam and initiation of replication by DnaA and ending the eclipse period. Under stringent conditions, RNA polymerase activity is altered by ppGpp and DksA and would not transcribe at the origin.
causing SeqA to remain bound, prolonging the eclipse period for as long as RNA polymerase remains bound to ppGpp.

This model requires the presence of a stringently controlled promoter upstream of oriC. In fact, genes do exist on either side of oriC, with gidA lying just upstream and mioC just downstream. It has been shown that transcription from the gidA promoter correlates with initiation of replication and that transcription from the mioC promoter correlates with the eclipse period, and both promoters are inhibited by ppGpp in vitro. (Ogawa and Okazaki 1994).

Another explanation could be the modification of transcription of genes controlling replication initiation. Down-regulation of dam during stringent arrest would cause GATC sites at oriC to remain hemi-methylated for long periods of time and would allow SeqA to bind indefinitely. Down-regulation of DnaA would simply turn off initiation, even in the absence of sequestration of oriC by SeqA. Up-regulation of SeqA is also a possibility, though it is unlikely based on the results of our experiment, as we have shown that over-expression of SeqA does not prolong the stringent response. In addition, it has been shown that constitutive expression of mioC inhibits initiation of replication in vivo (Su’etsugu 2003), and this would be another possible mechanism by which the cell could activate stringent arrest. It is also possible that many of these mechanisms work in conjunction during stringent arrest.

Possible Future Investigations:

Follow-up experiments testing the relationship between transcription at gidA and mioC and SeqA binding should be performed. Does transcription at gidA and mioC affect the stringent response? Do mutations in their promoters affect the stringent response?
Experiments could be performed to test levels of *seqA, dam, dnaA, gidA,* and *mioC* transcription under normal and stringent conditions, in an attempt to see if transcription at these genes is modified during stringent response. In addition, promoters at *gidA* and *mioC* could be deleted or replaced with non-stringent promoters to see if this would have any effect on stringent response.

Further experiments on stringent response release could also be useful. We display that over-expression of SeqA does not prolong the stringent response, but the experiment could be repeated with a *dam* over-expresser. If we could find a way to cause cells to remain arrested even after wash-out of SHX, we could come a lot closer to discerning the nature of the arrest mechanism.

**Conclusions:**

While the modification of transcriptional activity during the stringent response has been fairly well characterized, elucidating the nature of stringent arrest has been more elusive. This study provides very strong evidence that negative regulators of replication, SeqA in particular, are responsible for blocking initiation of replication during stringent arrest. In addition, changes in transcriptional activity brought about by ppGpp-bound RNA polymerase are both sufficient and necessary to cause stringent arrest.

It is still somewhat unclear as to exactly how a change in transcriptional pattern brings about arrest by SeqA at *oriC*. One model, which says that transcription at *oriC* serves to remove SeqA and end the eclipse period and that failure of RNA polymerase to transcribe at *oriC* during the stringent response causes SeqA to remain bound, is still valid, though other explanations are possible. More experiments such as the ones described above are necessary to determine the link between ppGpp and cell cycle arrest.
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